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Determination of the sulfur mustard hydrolysis product thiodiglycol by microcolumn liquid chromatography coupled on-line with sulfur flame photometric detection using large-volume injections and peak compression

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Abstract

A selective, direct and relatively rapid method has been developed for the determination of thiodiglycol (TDG) in aqueous samples. TDG is the main hydrolysis product of the chemical warfare agent sulfur mustard. The method of analysis is based on the on-line coupling of reversed-phase microcolumn liquid chromatography and sulfur-selective flame photometric detection. To improve sensitivity and efficiency, peak compression by displacement was used in combination with large-volume injections. A concentration of 1% *n*-propanol was added to the sample to obtain the best sensitivity and efficiency after a 10 μl injection. Detection limits of 0.25 μg/ml were achieved with efficiencies of $4 \cdot 10^5$ plates per meter. The method was successfully applied during the Fourth Official Proficiency Test organized by the Technical Secretariat of the Organization for Prohibition of Chemical Weapons for the determination of TDG in a soil sample.

Keywords: Warfare agents; Displacement chromatography; Peak compression; Large-volume injections; Detection, LC; Flame Photometric Detection, sulfur mode; Soil; Environmental analysis; Thiodiglycol; Sulfur compounds

1. Introduction

In recent years there has been a growing interest in the analysis of chemical warfare agents (CWAs) as well as their precursors, manufacturing byproducts and degradation products. This is mainly because of the coming into force of the Chemical Weapons Convention (CWC), which prohibits the development, production, stockpiling and use of CWAs. Analysis of CWAs and related compounds may play a key role in the verification of the treaty. Also, the destruction of existing stockpiled CWAs has to be carefully followed by analytical procedures. Recently an extensive review [1] was published which gives an overview of the state-of-the-art of the analytical methods used for these purposes and possible future trends.

Sulfur mustard [bis(2-chloroethyl)sulfide] is a CWA, which was frequently used during the First World War and more recently in the Iran–Iraq war

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[2]. Sulfur mustard is a vesicant, it affects the eyes and lungs and blisters the skin. In the environment, hydrolysis of sulfur mustard leads mainly to the formation of thiodiglycol [TDG, bis(2-hydroxyethyl)sulfide], a polar, stable and non-toxic compound. The destruction of sulfur mustard by hydrolysis or natural weathering may also result in the formation of TDG. Both compounds are included in the scheduled list of the CWC; sulfur mustard in Schedule-1 and TDG in Schedule-2 [3].

Gas chromatography–mass spectrometry (GC–MS) is the most commonly used method for the detection and identification of TDG in environmental samples [4–6]. GC–flame photometric detection (GC–FPD) is also used [7]. However, because TDG is a polar, neutral compound, direct GC-based methods are difficult and resulted in substantial tailing and broadening of the peak and unsatisfactory detection limits. Therefore, time-consuming sample preparation and derivatization prior to analysis are generally applied. The above time factor may become unacceptably prolonged, especially if many samples have to be analyzed. Besides, derivatization may lead to the formation of artefacts. Flow injection–MS and LC–MS [8–11] reduce the sample handling and derivatization requirements, and therefore increase the sample throughput. However, as well as with GC–MS, the high costs of such methods are a disadvantage. In other words, the development of rapid and sufficiently selective methods for screening purposes is desirable. The use of hyphenated spectroscopic techniques as GC–MS–MS and LC–MS–MS or NMR [12] methods for the unambiguous identification of the compounds can then be limited to suspect samples.

LC with electrochemical detection has been used for the direct detection of polar degradation and biodegradation products of sulfur mustard [13,14]. A relatively rapid and inexpensive method to screen aqueous samples for TDG and several other hydrolysis products of sulfur mustard is micellar electrokinetic chromatography with UV detection [15]. Detection of TDG can be achieved within seven minutes. However, the concentration detection limit for TDG is only 10 μg/ml and UV detection at 200 nm is not very selective, so analysis at low concentration levels in complex matrices will often be problematic. We developed a relatively rapid and selective screening method based on the on-line coupling of microcolumn liquid chromatography (micro-LC) with sulfur-selective flame photometric detection (S-FPD). Micro-LC–FPD as used in this paper was developed by Kientz et al. [16,17]. The interface principle is based on using a steep temperature gradient, at the tip of an introduction capillary, to generate a jet of column effluent droplets which are introduced into the detector flame. The micro-LC–FPD system has been used in the phosphorus mode for the determination of non-volatile organophosphorus acids such as alkyl methylphosphonic acids, which are degradation products of well-known nerve agents like VX, sarin and soman. The sensitivity of FPD in the sulfur mode is 10-fold lower compared with the phosphorus mode. To overcome the relatively poor sensitivity of S-FPD, large-volume injections and peak compression were investigated. Peak compression as used here is also called displacement LC and was applied for the enrichment of trace components in LC by Guiochon et al. [18]. Kientz et al. [19] used peak compression by displacement in micro-LC–FPD for the determination of alkyl methylphosphonic acids. The general characteristics and effects of displacement chromatography will be discussed under Results and discussion. In this paper, a suitable displacer was chosen and the concentration added to the sample was optimized for a maximized injection volume, and relevant analytical data were collected. The applicability of the optimized system was shown by the analysis of an aqueous extract of a soil sample.

2. Experimental

2.1. Materials

Analytical-grade formic acid, acetic acid, ammonium acetate, *n*-propanol and 2-propanol were purchased from Merck (Darmstadt, Germany). TDG and TDG sulfoxide were synthesized at the TNO–Prins Maurits Laboratory. Throughout the study, deionized water (Milli Q water purification system; Millipore, Milford, MA, USA) was used.

2.2. Instrumentation

The micro–LC–S-FPD system is shown schematically in Fig. 1. A Phoenix 20 CU (CE Instruments,

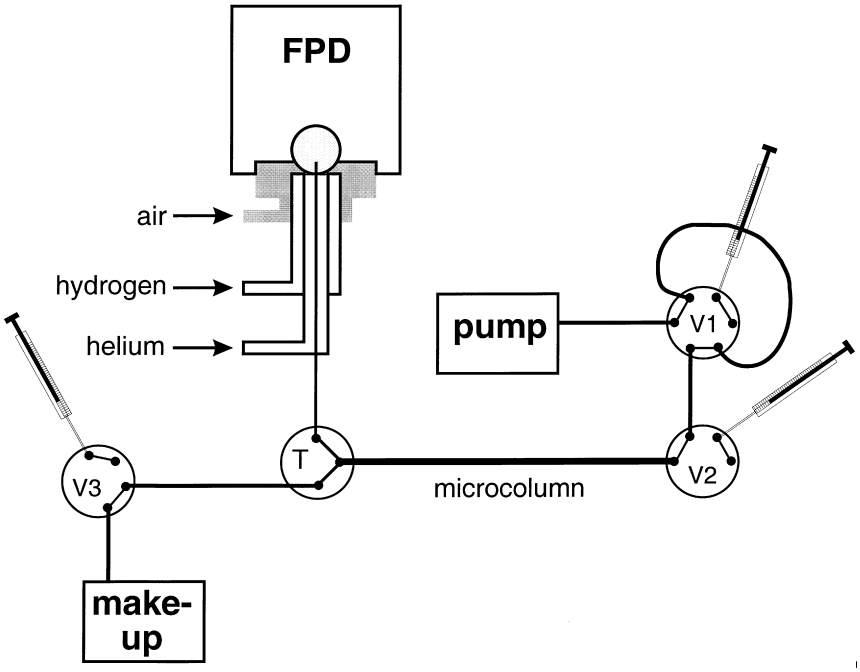

Fig. 1. Experimental micro-LC–S-FPD set-up. Column: LiChrosorb RP-18 15 cm×320 μm I.D.× 450 μm O.D., 5 μm particles; mobile phase: 50 m*M* ammonium acetate pH 4.0, 6 μl/min; gas flow-rates: hydrogen flow-rate, 600 ml/min; helium flow-rate, 40 ml/min; air flow-rate, 175 ml/min For further details, see Experimental section. The schematic has not been drawn to scale.

Milan, Italy) syringe pump was connected to a Valco (VICI, Schenkon, Switzerland) six-port valve (V1) with a variable external injection loop of polyether ether ketone (PEEK) tubing (Alltech, Breda, The Netherlands). This six-port valve was connected to a Valco microinjection valve with a 60 nl internal volume (V2). The fused-silica (Supelco, Bellefonte, PA, USA) column (15 cm×320 μm I.D.×450 μm O.D.) was slurry packed with 5 μm LiChrosorb RP-18 (Merck) bonded silica. The column outlet was inserted in a laboratory-made PEEK T-piece (T) together with the outlet of an optional Waters M590 make-up pump (Waters, Milford, MA, USA) via a Valco micro injection valve with a 60 nl internal volume (V3), allowing flow-injection optimization of the interface and detector. An introduction capillary (35 cm×100 μm I.D.×170 μm O.D.) passes from the T-piece through the interface into the flame of the FPD Model 380 (CE Instruments). The interface is described in detail elsewhere [16].

Micro-LC–UV experiments were performed using the same micro-LC system, but the LiChrosorb RP-18 column was glued to a piece of 75 μm I.D.×280 μm O.D. fused-silica (LC Packings, Amsterdam, The Netherlands) with a glass filter in between to retain the packing material. A window for on-column UV detection was made by burning off the capillary coating 4 cm from the outlet of the LC column. A Lauerlabs UV–Vis VWL (Prince Technologies, Emmen, The Netherlands) detector was used at 200 nm. The time constant was set at the lowest possible value of 0.02 s.

Micro-LC–electrospray mass spectrometry (micro-LC–ES-MS) experiments were carried out on a Quattro II triple-quadrupole instrument (Micromass, Altrincham, UK) with a standard ES interface. The instrument was operated in the positive ion mode and using a 30 V cone voltage. The scan range was m/z 55–300 at 1 scan/s. The mobile phase consisted of water with 0.2% formic acid and was provided at a flow-rate of 6 μl/min by a Waters M510 pump. The same microcolumn was used as in micro-LC–S-FPD.

2.3. Methods

To extract soil, ca. 10 g soil were put in an

Erlenmeyer flask, and water (10 ml) was added. After 10 min of ultrasonification the liquid fraction was decanted in a centrifuge tube. Another 10 ml water were added to the soil and after 10 min of ultrasonification decanted in another centrifuge tube. The extracts were centrifuged for 10 min at 10 000 rpm and afterwards combined.

All solvents and solutions were filtered prior to use over 0.45 μm pore size filter disks from Millipore. The LC injection volume was 10 μl except where indicated. All experiments were carried out at ambient temperature.

3. Results and discussion

3.1. Micro-LC–FPD system

Since we developed the on-line coupling of micro-LC and FPD [16,17], the detector has always been used in the phosphorus mode. So far S-FPD was not applied, because it has several drawbacks. One drawback is the relatively low sensitivity. Detection limits of 10 pg S/s are specified for the detector used, compared to 1 pg P/s.

A main disadvantage of FPD is the quenching of the sulfur chemiluminescence by organic compounds. This quenching is probably caused by collisions of analyte molecules with molecules of coeluting organic compounds. Collision-induced quenching will reduce the sensitivity with the degree of quenching being dependent on the mass flow of organic molecules [20]. In GC, this phenomenon can reduce the sensitivity of analytes eluting on the tail of the solvent peak or not-baseline separated from other analytes. In LC the situation can become much more serious, especially in the case of reversed-phase LC when the effect of the organic modifier has to be considered. The amount of organic modifier has to be minimized to obtain satisfactory detection limits. Therefore, an LC system with an aqueous eluent is preferred. An acceptable capacity factor, k', of 2.4 was obtained for TDG using a LiChrosorb RP-18 column with an aqueous eluent containing 50 m*M* ammonium acetate adjusted to pH 4.0 with acetic acid. The hold-up time to calculate k' was determined by the injection of TDG sulfoxide. Acidic conditions were applied to prevent silica dissolution at higher pH values, which will cause baseline instability, and even clogging of the introduction capillary. Ammonium acetate was chosen as the buffering component because of its volatility. Non-volatile buffer salts can also cause introduction instability and clogging of the introduction capillary to the FPD system. The eluent flow-rate was set at 6 μl/min At this flow-rate the interface operation is stable without the need of a make-up flow [16].

3.2. Optimization of gas flow-rates

As in phosphorus-selective micro-LC–FPD [16,17] and in the recently developed capillary electrophoresis–FPD system [21,22], the hydrogen flow-rate cannot be varied because it provides the heat gradient and determines the position of the detector flame necessary to induce a stable jet of droplets. In all experiments, the hydrogen flow-rate was set at 600 ml/min. Helium flow-rates up to 50 ml/min did not affect either the response or the noise level. However, higher flow-rates resulted in system instability (cf. [16]). To be on the safe side, the helium flow-rate was set at 40 ml/min. The dependence of the response on the air flow-rate was studied in the 130–465 ml/min range (Fig. 2). The optimum was found at 175 ml/min. This flow-rate was used for all further experiments.

3.3. Optimization of peak compression and large-volume injections

As mentioned before, the S-FPD is relatively insensitive. Conventional 60 nl injections resulted in absolute detection limits of 5 ng TDG. This means that the best concentration detection limits that could be achieved, were approximately 100 μg/ml, which is much too high for our area of application. To make the method useful for screening purposes, detection limits of at least 1 μg/ml are needed. A drawback of using an aqueous eluent and a reversed-phase column for the determination of TDG in aqueous samples is that on-column focusing of TDG was found to be impossible because of the high polarity of both TDG and the eluent. That is, the relatively simple concentration of the analyte by means of large-volume injections and subsequent on-column focusing could not be used.

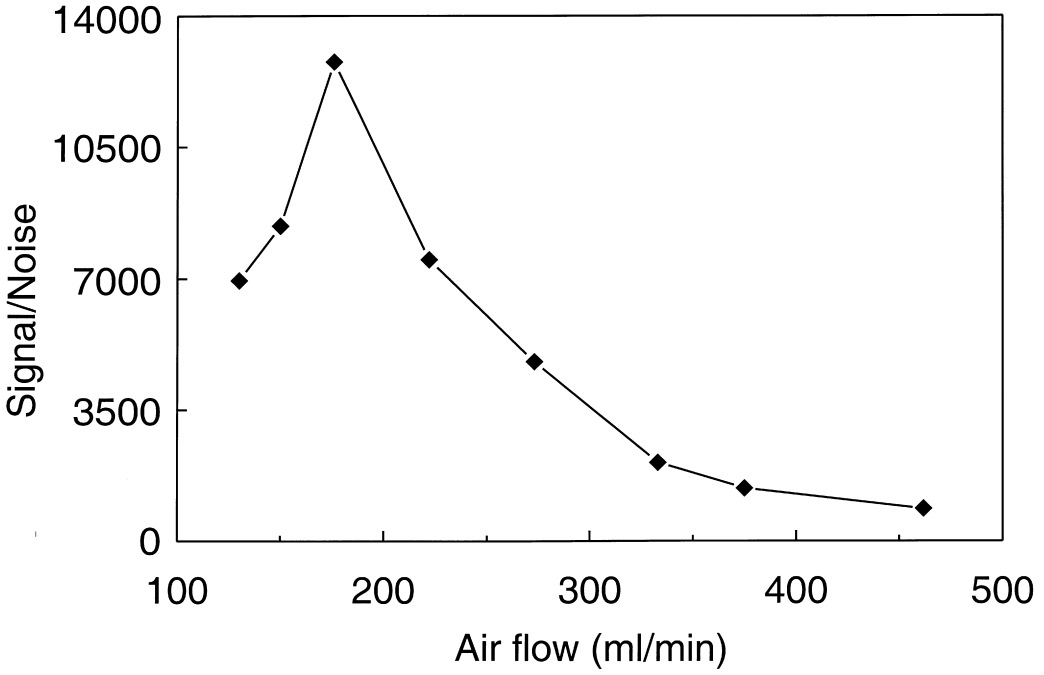

Fig. 2. Influence of the air flow-rate on the signal-to-noise ratio of TDG. Injection volume, 60 nl; concentration, 10 mg/ml; hydrogen flow-rate, 600 ml/min; helium flow-rate, 40 ml/min.

In order to enhance the analyte detectability, peak compression was attempted. In displacement chromatography, an analyte can selectively be compressed to an extremely narrow band by adding a displacer to the sample. In order for this to be successful, the displacer should have a capacity factor slightly larger than the capacity factor of the analyte to be compressed. In addition, the sample size and concentration of the displacer must be large enough for the non-linear displacement effect to occur. This effect pushes the analyte band forward and at the same time significantly increases its concentration and reduces its bandwidth. The loadability of the column is also considerably increased, making large-volume injections possible. The effect can be considered as a local gradient of up to 100% organic modifier at a specific position in the chromatogram. Theory shows that the apparent column efficiency may exceed the conventional column efficiency by up to two orders of magnitude [18]. At the same time the analyte concentration in the band may be increased more than 10-fold. Together with the increased loadability of the column, this may help to improve detection limits by at least two orders of magnitude. The increased concentration in a compressed peak is especially convenient when on-line S-FPD is used because of the quadratic response factor of the sulfur chemiluminescence. In order to use displacement chromatography in the determination of TDG, a suitable displacer had to be selected, and its concentration and the injection volume had to be optimized.

3.4. Displacer

An alcohol was selected as the displacer for the determination of TDG. Alcohols, which are common modifiers in LC, do not contain any other element, such as phosphorus, that may give a response in S-FPD at the high concentrations typically used in displacement LC. More importantly, the polarity and, consequently, the capacity factor of small alcohols are relatively close to those of TDG. As stated above, the optimal displacer is an alcohol with a slightly higher capacity factor than TDG. However, the solubility of the alcohol in water has to be large

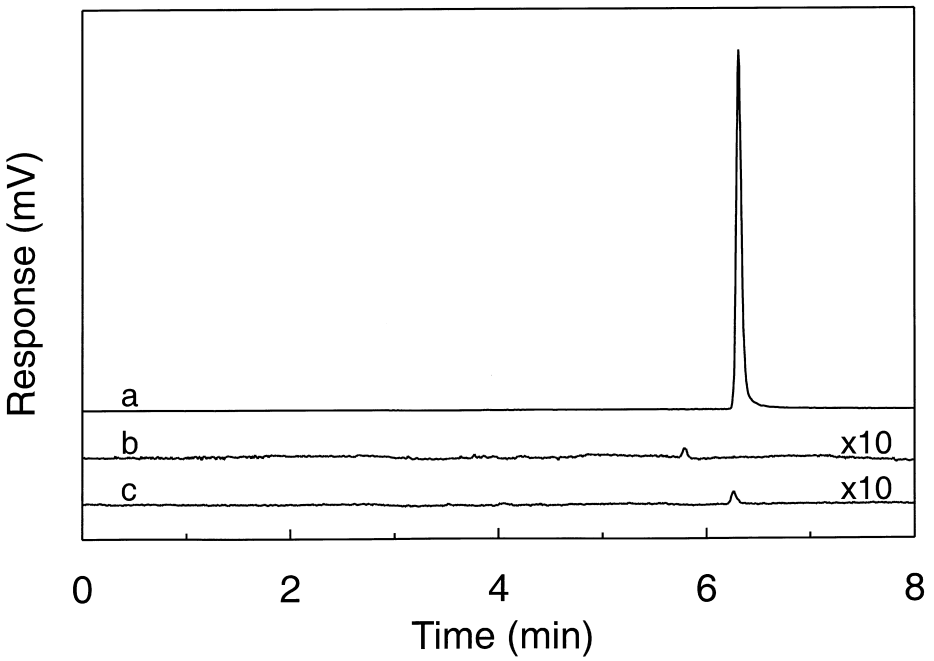

Fig. 3. Micro-LC–S-FPD chromatograms of 10 μg/ml TDG compressed by (a) 1% *n*-propanol, (b) 1% 2-propanol and (c) 0.5% 2-propanol.

enough which implies that propanols are an obvious first choice. Fig. 3 shows chromatograms obtained after the addition of 1% *n*-propanol (trace a) and 1% or 0.5% 2-propanol (traces b and c, respectively) to a 10 μg/ml solution of TDG in water. The strongest displacement effect was observed with 2-propanol, as can be concluded from the shorter retention time of TDG. However, this modifier is seen to cause a dramatic loss of sensitivity. Probably, this is mainly caused by the large amount of TDG extracted by the 2-propanol plug as compared with the *n*-propanol plug. The TDG dissolved in the propanol plug can hardly be detected in the FPD because of both dilution of TDG in the plug and quenching by propanol. Obviously, *n*-propanol is the better displacer for TDG, even though its capacity factor is slightly higher.

3.5. Concentration of n-propanol and injection volume

When optimizing the percentage of *n*-propanol added to the sample and maximizing the injection volume, the principal objective is to increase the sensitivity of the sulfur-selective determination of TDG. However, the separation efficiency, selectivity and repeatability of the procedure are also important factors. The injection volumes tested were 2.5, 5, 10 and 20 μl, and the concentrations of *n*-propanol added to the sample were varied from 0 to 3% (v/v). The concentration of TDG after the addition of *n*-propanol was 10 μg/ml in all samples. Fig. 4 shows the overlay of chromatograms obtained after 10 μl injections of samples containing eight different concentrations of *n*-propanol. Clearly, a dramatic increase of sensitivity as well as efficiency is obtained upon addition of the alcohol. In its absence 10 μg/ml TDG is below the detection limit. Additional micro-LC–UV experiments confirmed that, without a displacer, TDG moves through the column as a plug. The width of the plug is approximately 10 μl, the same as the injection volume. Or, in other words, there is indeed no on-column focusing effect because of the high polarity of both the analyte and the eluent. In the presence of 0.25% *n*-propanol, a broad band can be observed: the displacer starts to push the analyte through the column. Increasing the concentration of the displacer is seen to result in a more

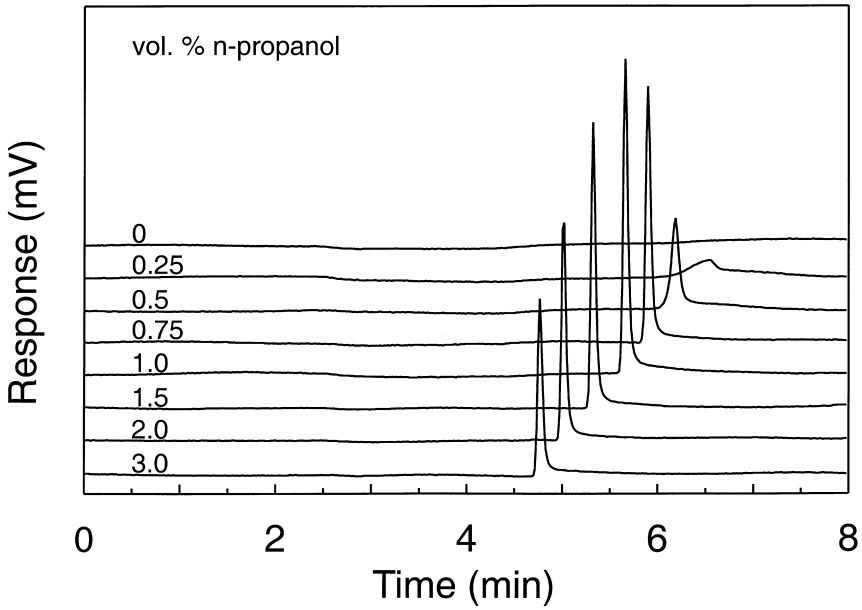

Fig. 4. Micro-LC–S-FPD chromatograms obtained after the addition of various percentages of *n*-propanol to a TDG sample. Final TDG concentration, 10 μg/ml.

regular peak shape although some peak deformation can be observed, which is characteristic of the L-shape profiles of strongly displaced component bands in non-linear chromatography [18].

The effect of the amount of displacer on the three most important values; peak height, efficiency and retention time, can be read from Fig. 5A–C, respectively. Both peak height and efficiency reach a maximum value at about 1% *n*-propanol added. Further increase of the displacer concentration causes a slight decrease of sensitivity. Additional micro-LC–UV experiments showed the peak height to continually increase over the whole concentration range investigated. This suggests that quenching probably starts to be significant when the concentration of *n*-propanol is larger than 1%. That this may well be true is shown by the micro-LC–ES-MS analysis of a 10 μl injection of 10 μg/ml TDG in 1% *n*-propanol, depicted in Fig. 6A. The upper trace of the selected ions of TDG (*m/z* 105+123, $[M+H-H_2O]^+$ and $[M+H]^+$) and the lower trace of the selected ions of *n*-propanol (*m/z* 61+121, $[M+H]^+$ and $[2M+H]^+$) clearly show that TDG elutes on the very slope of the *n*-propanol front. This adds to the probability that quenching may play a significant role when the displacer concentration is relatively high. Besides, the micro-LC–ES-MS analysis clearly shows that a significant part of TDG is dissolved in the *n*-propanol plug. This becomes clear from the peak shape of TDG shown in Fig. 6A. The peak deformation has an identical shape as that of the *n*-propanol plug of Fig. 6B. In other words, at the interface of the eluent and the *n*-propanol plug, TDG is distributed between both phases.

The maximum efficiency achieved at 1% *n*-propanol is $4 \cdot 10^5$ plates/m (Fig. 5B), which is a 25-fold increase compared with the plate number of $1.5 \cdot 10^4$ obtained after injection of 60 nl of 1 mg/ml TDG in water. The column efficiency slowly decreases when the displacer concentration exceeds 1%. This is because the peak width remains approximately unchanged (3.2 s) while the retention time decreases at increasing displacer concentration. Nilsson and coworkers [23,24] reported similar observations, although their peak compression method was different from the one used here. They achieved peak compression by reducing the elution strength in an induced system peak. The analytes then slow down and will be concentrated in front of the system peak.

After the elution of TDG, a certain delay time is

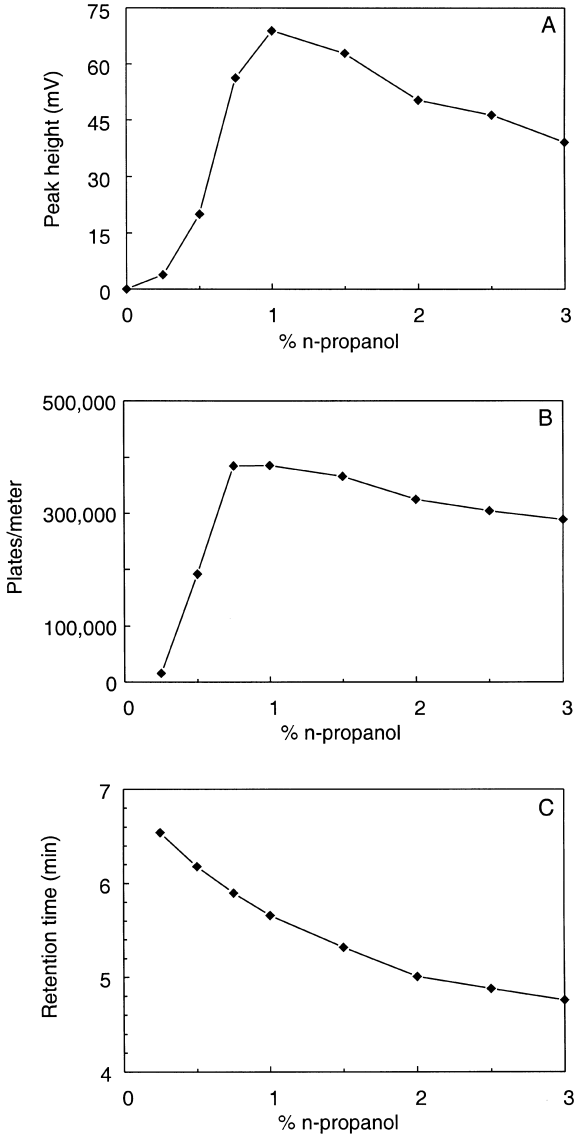

Fig. 5. Optimization of percentage *n*-propanol added to the sample with regard to (A) peak height (mV), (B) efficiency (plates/m) and (C) retention time (min). Chromatograms are displayed in Fig. 4.

necessary before the next injection can be made because all of the displacer has to be eluted from the column. The time interval between two injections has to be 10 min, as can be read from the micro-LC–ES-MS selected ion trace of Fig. 6B. When an unknown sample is injected, an extra step will be needed to remove any strongly retained sample components from the column. Usually, a 10 μl injection of pure methanol directly after the elution of TDG is sufficient. In that case the time interval between injections should be about 12 min, to ensure that the entire methanol plug will have been eluted.

As regards Fig. 5C, according to expectations, the retention time decreases with an increasing concentration of displacer. At the optimum value of 1% *n*-propanol, some retention is still left: the capacity factor is reduced from 2.4 under isocratic conditions (60 nl of 1 mg/ml TDG) to 0.8 under displacement conditions (10 μl of 10 μg/ml TDG in 1% *n*-propanol).

The injection volume of 10 μl was found to be close to the maximum value. If higher injection volumes were used the maximum sensitivity and efficiency were for conditions which virtually reduced the capacity factor to zero. For the rest, the maximum sensitivity was only slightly better than reported above, because quenching effects were observed to increasingly play a role at larger injection volumes. Besides, a significant part of TDG becomes dissolved in the *n*-propanol plug.

3.6. Background sulfur doping

From the literature it is known that sulfur doping (intentional or unintentional) of the detector flame of an FPD system affects its performance [25]. There are three reported advantages of sulfur doping by adding e.g. SO_2: (i) improved detection limits, (ii) linear response vs. analyte sulfur concentration in the lower concentration range, and (iii) the possibility of indirect detection by quenching of the background sulfur. In order to study the potential of sulfur doping for improved detection in micro-LC–S-FPD, different concentrations of TDG (1–20 μg/ml) in water were added to the detector via the make-up delivery pump at a flow-rate of 5 μl/min (cf. Fig. 1). That is, TDG was used as the background sulfur source. At each concentration 10 μl of 1 μg/ml TDG in 1% *n*-propanol was injected and the peak height was measured. As the best result, the peak height became twice as high, viz. when adding 10 μg/ml TDG as the make-up solution. Because of the quadratic nature of the response, this means that the analyte detectability was improved about 1.4-fold.

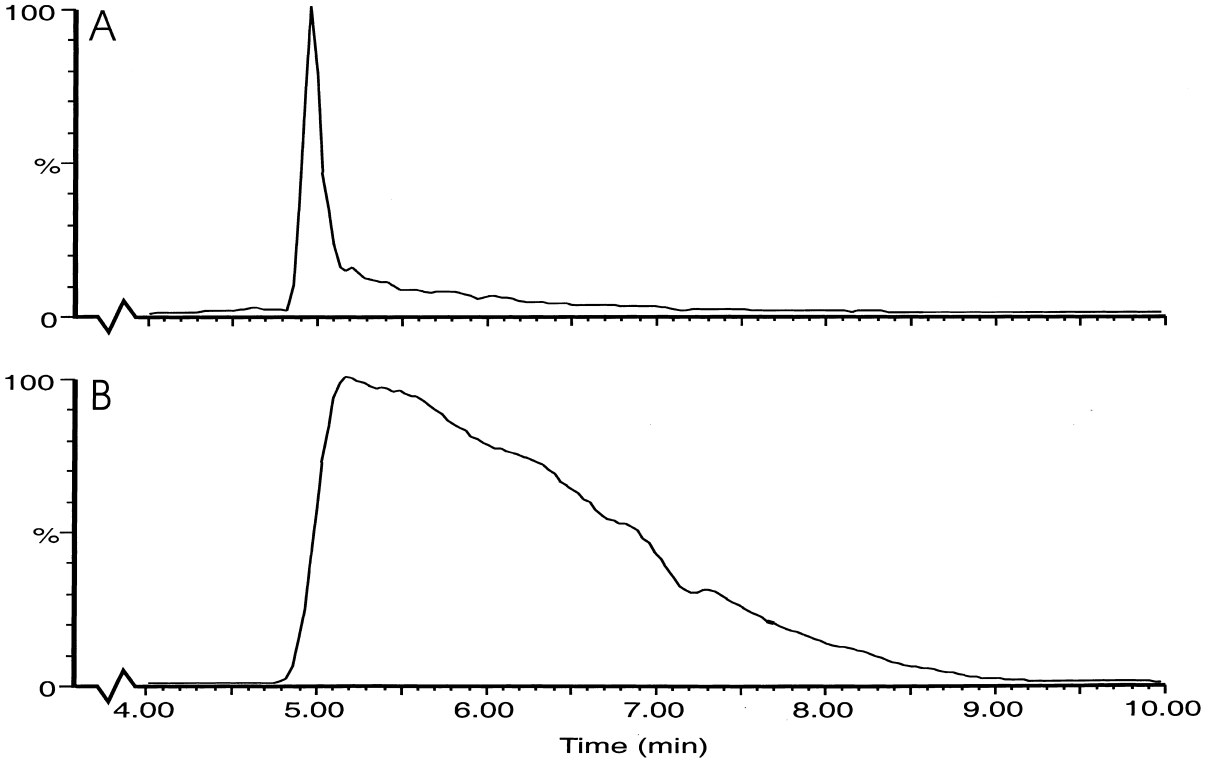

Fig. 6. Micro-LC–ES-MS of 10 μg/ml TDG in 1% *n*-propanol. Selected ion chromatogram of (a) *m/z* 105+123, $[M+H\text{-}H_2O]^+$ and $[M+H]^+$, of TDG, and (b) *m/z* 61+121, $[M+H]^+$ and $[2M+H]^+$, of *n*-propanol.

Because of this minor effect, sulfur doping was not applied during further experiments.

3.7. Detection limit, linearity and repeatability

Fig. 7 shows a micro-LC–FPD chromatogram of a 10 μl injection of 0.25 μg/ml TDG in 1% *n*-propanol. This concentration can be considered as the detection limit of the present method.

A somewhat notorious characteristic of the S-FPD is its non-linear response. The response model is generally taken to be $I \propto [S]^n$, where I is the sulfur emission intensity, and S the sulfur concentration. The value of *n* is predicted to be 2. However, in practice values of *n* in the range from one to slightly over two are reported. Reasons cited for such deviations from the theoretical value of two include non-optimum flame conditions, compound-dependent decomposition, competitive flame reactions and quenching effects [25].

A calibration curve for 10 μl injections of TDG in 1% *n*-propanol (11 data points) was constructed using peak compression and large-volume injections. For the first nine points, from the detection limit up to 20 μg/ml, a good fit ($r^2 > 0.9999$) is obtained by using the equation $y = ax^n$ ($a = 1.17 \pm 0.04$, $n = 2.40 \pm 0.01$). However, the value found for *n* is rather high compared with literature values (cf. above). At TDG concentrations of over 20 μg/ml the response started to fall off. Most probably, this is due to increased quenching at high analyte concentrations: since quenching is also quadratic it starts to take effect at higher concentrations of sulfur. This agrees with GC–S-FPD results reported elsewhere [25].

The repeatability of the procedure was tested by ten consecutive 10 μl injections of 10 μg/ml TDG in 1% *n*-propanol. The time interval between injections was 10 min. The relative standard deviations were 1.7% for the retention time, 2.7% and 3.5% for the peak area and peak height, respectively and 3.5%

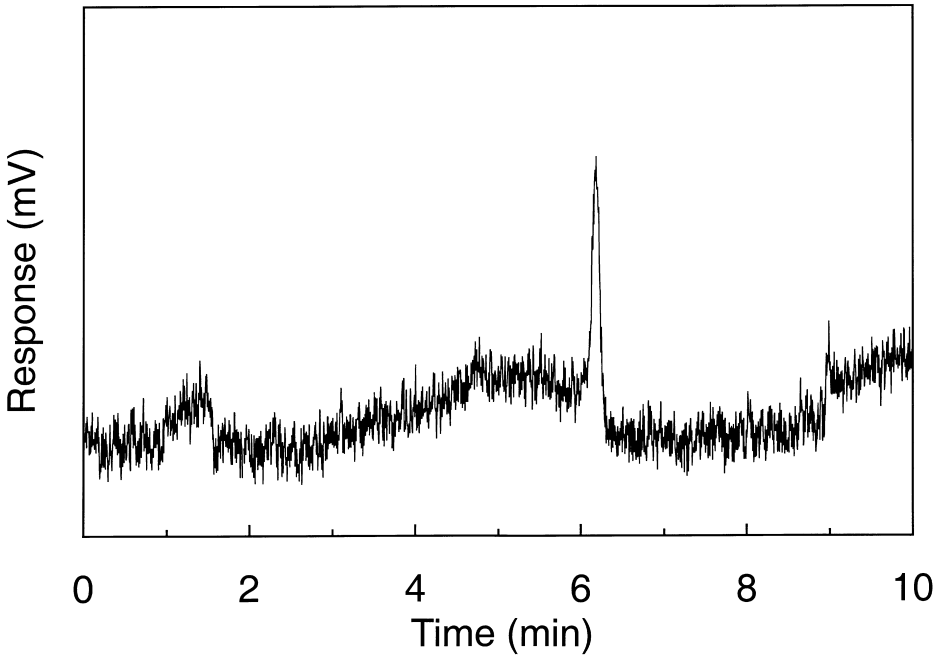

Fig. 7. Micro-LC–S-FPD chromatoram of a 10 μl injection of 0.25 μg/ml TDG in 1% *n*-propanol.

for the efficiency (plates/m). These can be called highly satisfactory results.

3.8. Application

Micro-LC–S-FPD was used to screen samples for the presence of TDG during the Fourth Official Proficiency Test organized by the Technical Secretariat of the Organization for Prohibition of Chemical Weapons (OPCW). Corresponding blank samples were provided for all test samples. A soil sample from a waste pit and a blank soil of the similar type taken close to that waste pit were extracted with water according to the standard operation procedure [26]. After extraction 1% *n*-propanol was added to both extracts. Fig. 8 shows the resulting chromatograms. A small peak was observed in the blank, which was absent in the sample. This peak could not be attributed to TDG because of the too small retention time. One prominent sulfur-containing peak is seen to occur in the sample extract, but not in the blank. From its retention time, and the extremely high efficiency, the peak could tentatively be assigned to TDG. This conclusion could be drawn less than 10 min after extraction of the soil sample had been completed which is, of course, highly advantageous for the goal in mind, rapid screening. The TDG concentration was calculated to be 10 μg/ml after standard addition of the sample extract with 5 μg/ml TDG. This concentration corresponded with a spiking level of approximately 20 μg/g TDG of the soil sample. Subsequently, the identification was confirmed by NMR and LC–ES-MS analysis and by GC–MS after trimethylsilyl derivatization.

4. Conclusions

The on-line coupling of reversed-phase micro-LC and S-FPD provides a selective and relatively rapid procedure for screening aqueous samples for the presence of TDG. The combined effect of peak compression by displacement with *n*-propanol and large-volume injections (10 μl) resulted in detection limits of 0.25 μg/ml TDG with a total analysis time of 12 min. The separation efficiency is extremely high ($4 \cdot 10^5$ plates/m). The potential of the method

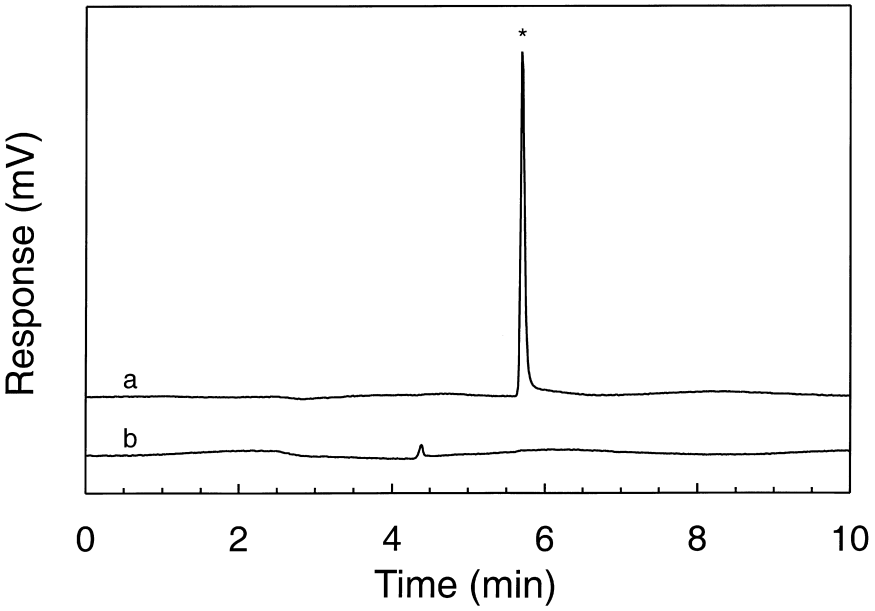

Fig. 8. Micro-LC–S-FPD of an aqueous extract of a soil sample. (a) Soil sample extract; (b) blank soil sample extract. Injection volume, 10 μl; 1% *n*-propanol added to extract. For further conditions, see text.

was demonstrated by analyzing a soil sample during an OPCW Proficiency test.

Future research will be focused on modification of the procedure to enable the determination of sulfur-containing hydrolysis products of other sulfur mustard agents in aqueous samples.

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